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Correlation between Extracellular Fibrils and Attachment of *Rhizobium leguminosarum* to Pea Root Hair Tips

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As part of a project meant to characterize molecules involved in nodulation, a semiquantitative microscopic assay was developed for measuring attachment of *Rhizobium leguminosarum* cells to pea root hair tips, i.e., the site at which *R. leguminosarum* initiates nodulation. This form of attachment, designated as cap formation, was dependent on the incubation pH and growth phase, with optimal attachment at pH 7.5 and with bacteria in the early stationary phase of growth. Addition of glucose to the growth medium delayed the initiation of the stationary phase and cap formation, suggesting a correlation between cap formation and carbon limitation. Attachment of *R. leguminosarum* was not inhibited by pea lectin haptens which makes it unlikely that lectins are involved under the tested conditions. Moreover, heterologous fast-growing rhizobia adhered equally well to pea root hair tips. Since the attachment characteristics of a Sym plasmid-cured derivative were indistinguishable from those of the wild-type strain, the Sym plasmidborne nodulation genes are not necessary for attachment. Sodium chloride and various other salts abolished attachment when present during the attachment assay in final concentrations of 100 mM. *R. leguminosarum* produced extracellular fibrils. A positive correlation between the percentage of fibrillated cells and the ability of the bacteria to form caps and to adhere to glass and erythrocytes was observed under various conditions, suggesting that these fibrils play a role in attachment of the bacteria to pea root hair tips, to glass, and to erythrocytes.

The gram-negative soil bacterium *Rhizobium* species attaches to the root hair tips of leguminous plants as a first step in the infection process leading to a nitrogen-fixing symbiosis. In fast-growing rhizobia the nodulation genes are located on a large plasmid, the so-called Sym plasmid. The molecular basis of rhizobial attachment is still not clear. Several investigators reported host-plant lectins to be specifically involved in attachment (2, 4, 23, 31). Attachment studies of *Rhizobium trifolii* to clover root hairs fit within this lectin recognition theory (4, 31). These studies showed that heterologous rhizobia as well as Sym plasmid-cured *R. trifolii* adhered only weakly to clover root hairs in comparison with *R. trifolii* wild-type strains. However, a number of other studies suggested that attachment of rhizobia is not a host-specific process and is not mediated by lectins (1, 21).

Hardly anything is known about the molecular nature of bacterial factors involved in the attachment process, but roles for lectin receptors, cellulose fibrils, and fimbriae have been proposed (5, 12). Lectin receptors have been found in capsular polysaccharides, extracellular polysaccharides, and lipopolysaccharide of the rhizobia (12, 20, 27, 29). Their occurrence is a prerequisite in the theory of lectin-mediated attachment. Cellulose fibrils, which are produced by many rhizobia (6, 17) might also be involved in the attachment process as a second step (5). Analogous results were found for the attachment process of the closely related bacterium *Agrobacterium tumefaciens* (15). Since proteinaceous filamentous fimbriae play an important role in the attachment of various enterobacteriaceae to their host cells (see reference 7 for a review; T. H. Korhonen, M. Rhen, V. Väisänen-Rhen, and A. Pere, *in* D. E. S. Stewart-Tull, ed., *Immunology of the bacterial cell envelope*, in press) and in the association of *Klebsiella* spp. with grass roots (8), an involvement of fimbriae in rhizobial attachment cannot be excluded. Several rhizobial strains have been shown to possess fimbriae. Heumann (10) qualified the polarly exposed structures of the star-forming *Rhizobium lupini* as fimbriae. Tsien (24) reported that *Rhizobium japonicum*, *Rhizobium phaseoli*, and "cowpea" *Rhizobium* spp. have fimbriae which are polarly exposed on the cell surface. Stemmer and Sequeira (Abstr. Annu. Meet. Am. Phytopathol. Soc. 1981, no. 328) were able to visualize fimbriae of *R. japonicum*, *R. trifolii*, and *Rhizobium meliloti* after cultivating these strains under special conditions.

As part of a program in our laboratory aimed to characterize factors involved in nodulation by *Rhizobium leguminosarum* at the molecular level, we initiated the present study. Since attachment leading to nodulation starts at the root hair tip, we could not use a number of previously described attachment assays (e.g., see reference 18) in which the number of attached bacteria was quantified by counting radiolabeled bacteria or by counting CFU from root segments, with no regard for the site at which the bacteria adhered. Therefore we developed a semiquantitative attachment assay which enabled us to quantify attachment of the rhizobia at the site of infection, i.e., the developing root hair (see also reference 31). Using this assay we observed a positive correlation between the degree of fibrillation of *R. leguminosarum* cells and the ability to attach to pea root hair tips. Optimal attachment ability was induced by carbon limitation. The results do not support the lectin recognition theory.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *R. leguminosarum* 248, harboring Sym plasmid pRL1JI, is able to nodulate and fix N_2 on peas. Its Sym plasmid-cured derivative 248^c is $Nod^- Fix^-$. *R. leguminosarum* RBL1 is $Nod^+ Fix^+$ on peas. *R. trifolii* 5020 is $Nod^+ Fix^+$ on clover. Its Sym plasmid-cured derivative 5039 is $Nod^- Fix^-$. *R. trifolii* 5523 (strain 5039 harboring the *R. leguminosarum* Sym plasmid pRL1JI) is $Nod^+ Fix^+$ on peas. *R. trifolii* 0403, kindly provided by

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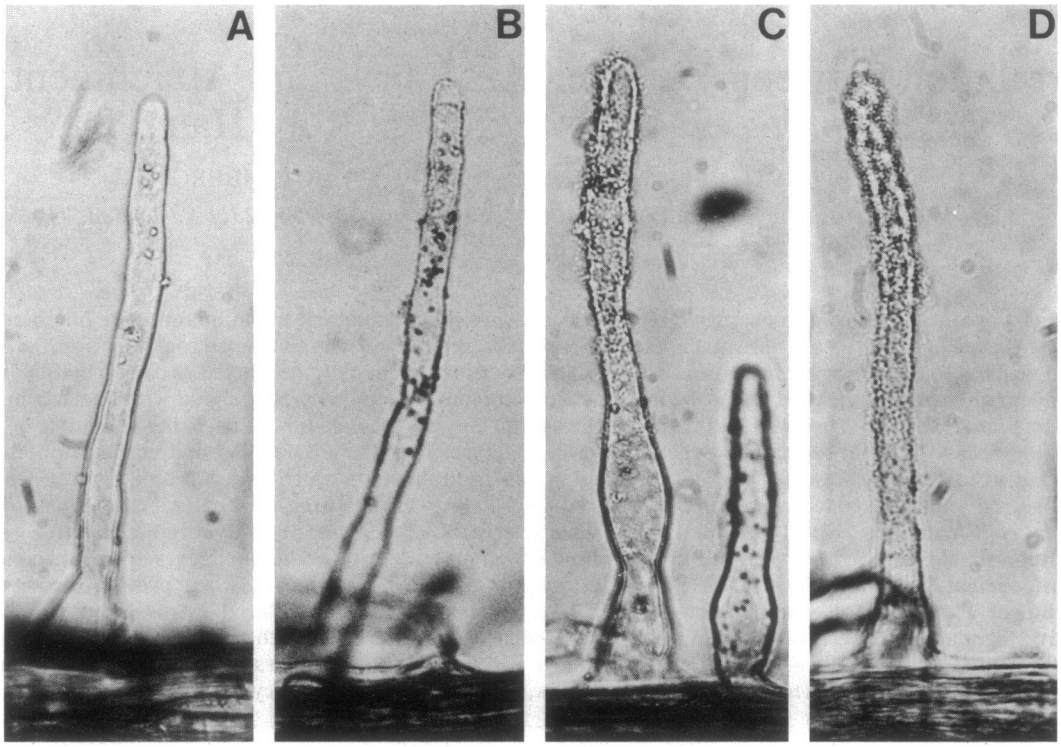

FIG. 1. Phase-contrast photographs of the four classes of attachment of rhizobia to pea root hair tips. (A) Class 1, no attached bacteria; (B) class 2, few attached bacteria; (C) class 3, the apical portion of the root hair covered with bacteria; (D) class 4, many attached bacteria forming a caplike structure on top of the root hair. Magnification, ×400. Conditions for the assay were as described in the Materials and Methods.

F. B. Dazzo, is Nod^+ Fix^+ on clover. *R. phaseoli* 1233 and RBL25 are Nod^+ Fix^+ on *Phaseolus* beans. *R. meliloti* 1021 and LPR2 are Nod^+ Fix^+ on *Medicago* sp. *R. lupini* M2 is Nod^+ Fix^+ on lupin. *R. japonicum* 784 and RBL25 are Nod^+Fix^+ on soybeans. All *nod*$^+$ *fix*$^+$ strains are Nod^- Fix^- on heterologous host plants. A^+ medium contains (per liter of deionized water): yeast extract (Difco Laboratories, Detroit, Mich.), 0.8 g; glucose, 10.0 g; mannitol, 3.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; KH_2PO_4, 0.993 g, and K_2HPO_4, 0.318 g. TY medium contains (per liter): tryptone (Difco), 5.0 g; yeast extract (Difco), 3.0 g; and $CaCl_2 \cdot 2H_2O$, 1.0 g.

Bacteria were maintained on slopes with solid A^+ medium at 4°C. Bacteria for attachment assays were cultivated at 28°C in 100-ml Erlenmeyer flasks containing 50 ml of TY medium under vigorous aeration (180 rpm). To study pellicle formation, we cultivated the bacteria under the same conditions except that shaking was omitted. Growth was monitored either by measuring A_{620} with a Vitatron colorimeter or by direct cell counting with a hemacytometer.

Plants. Pea seeds (*Pisum sativum* cv. Rondo) were obtained from Cebeco, Rotterdam, The Netherlands. Seeds were surface sterilized by treatment with 98% sulfuric acid for 10 min followed by five successive washings with sterile deionized water. Subsequently they were incubated for 10 min in a 10% sodium hypochlorite solution, commercial grade, washed extensively with sterile water, preswollen in water for 15 to 18 h, and allowed to grow in a growth chamber for 6 to 8 days in coarse gravel soaked in nitrogen-free medium (22).

Attachment assay. After determining the A_{620}, we centrifuged a volume of 1 to 5 ml of bacterial suspension in an Eppendorf centrifuge for 25 s at maximum speed. The pellet was suspended in 25 mM phosphate buffer (pH 7.5 [unless stated otherwise]) to a final A_{620} of 0.070, which corresponds to 1.5×10^8 to 2.0×10^8 bacteria per ml. Three to five lateral pea seedling roots, approximately 2.5 cm in length, were incubated in 5 ml of bacterial suspension for up to 2 h at room temperature under gentle agitation on a rotary table (2 rpm). After incubation, the roots were washed 10 times by vigorous shaking in phosphate buffer to remove nonattached and weakly attached bacteria and placed on a microscope slide. Attachment was quantified by randomly screening at least 100 root hairs in the zone of developing root hairs with a phase-contrast microscope (400-fold magnification). Attachment to root hairs was distinguished into four classes (see Fig. 1): class 1, no attached bacteria; class 2, few attached bacteria; class 3, the apical portion of the root hair covered with bacteria; class 4, many attached bacteria forming a caplike aggregate on top of the root hair. The percentage of root hairs of each class was calculated. Class 4 was chosen to represent optimal tip attachment. Inhibition of attachment by salts or by saccharides was tested by immersion of the seedlings in the bacterial suspension supplemented with these compounds. Saccharides were tested in a final concentration of 50 mM.

Determination of percentage of fibrillated cells. Samples of bacterial cultures were placed on pioloform (Wacker Chemie, Munich)-coated grids. The grids were air dried at room temperature and negatively stained with a 1% phosphotungstic acid solution (pH 7.2) for 5 min. Excess liquid was carefully removed before final air drying. Observations were made with a Philips EM300 electron microscope operating at 60 kV. The percentage of fibrillated cells was

estimated by examining 50 randomly selected cells. Only unclumped cells were counted since it could not be judged which of the cells located in a clump were fibrillated.

Hemagglutination. Hemagglutination was studied with human, calf, horse, and guinea pig erythrocytes, each type washed three times and diluted to a 2% suspension in 25 mM phosphate-buffered saline, pH 7.5. Titrations were carried out in microtiter plates. Bacteria were suspended in phosphate-buffered saline in a doubling-dilution series before the erythrocytes were added. Hemagglutination was carried out at room temperature and took at least 5 to 6 h. Mannose was added to a final concentration of 50 mM in a test for mannose-resistant hemagglutination.

RESULTS

Conditions for attachment to root hair tips. Cells of *R. leguminosarum* 248 attached to the developing root hairs of pea seedlings, and clumps of bacteria, designated as caps, formed at the tips of the root hairs (Fig. 1D). The variability of the test was about 5% and depended largely on the condition of the roots. Only very few bacteria adhered to epidermal cells under the experimental conditions used. The bacteria also adhered to wound tissue and dead epidermal cells. In a time course study (Fig. 2) caps were not observed during the first 30 min. A rapid increase in the number of caps was observed after approximately 40 min, and a maximal level was reached after 60 to 90 min. Therefore, a standard incubation time of 120 min was chosen. A minimal number of bacteria was necessary to obtain caps within the time of incubation. When the concentration of bacteria was less than 10^7/ml, only a small number of caps (<10%) was observed after 2 h. Therefore, 1.5×10^8 to 2.0×10^8 bacteria per ml were used for the experiments. Optimal attachment occurred at pH 7.5, and virtually no caps were observed at pH < 6. Attachment was strongly dependent on the growth phase of the bacteria. Bacteria in the lag phase showed moderate attachment ability, whereas bacteria in the early to mid-log phase showed a weak adherence. Optimal attachment was observed during the late log to early stationary phase (Fig. 3). Similar results were found for *R. leguminosarum* RBL1. Optimal attachment coincided with agglutination of the cells to glass as judged from the clearly visible ring of clumped cells on the glass wall of the Erlenmeyer flask at the liquid-air interphase.

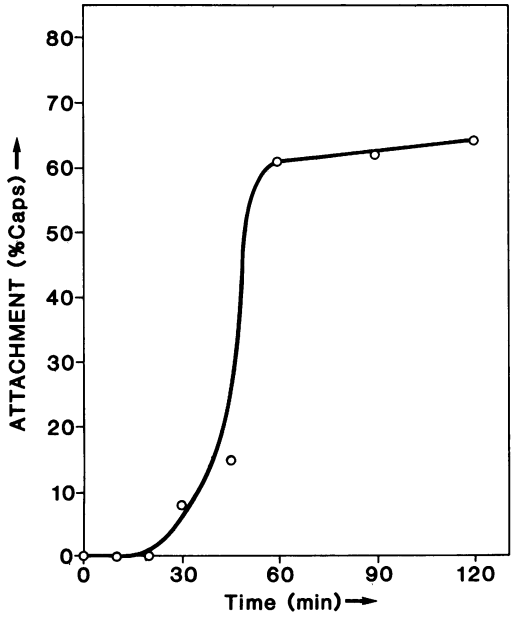

FIG. 2. Time course of cap formation (class 4 attachment) on pea root hair tips by *R. leguminosarum* 248 cells. The cells were harvested at an A_{620} value of 0.70. For further details, see the legend to Fig. 1.

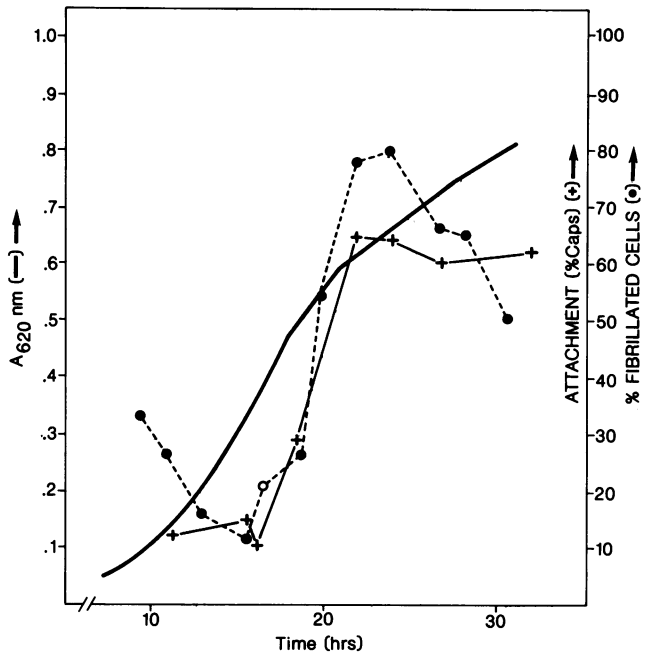

FIG. 3. Cap formation and fibrillation of *R. leguminosarum* 248 cells during growth in batch culture. Bacteria were harvested at several A_{620} values and added to the pea roots in a final concentration of 1.5×10^8 to 2.0×10^8 cells per ml. Attachment was measured after 2 h of incubation. The percentage of fibrillated cells was estimated by electron microscopy. For further details see Materials and Methods.

Growth limitation. After the initiation of agglutination to the glass the number of bacteria remained constant, suggesting limitation for one of the nutrients (see also reference 14). Since the addition of glucose to a final concentration of 1 mM caused a shift of the initiation of agglutination to glass to a higher A_{620} value, limitation for a carbon source in this medium apparently coincides with agglutination. Similarly, addition of glucose to 2 mM shifted the time of agglutination to glass to an even higher A_{620} value. Cell counting showed that the number of bacteria increased with the addition of glucose (Table 1). When the attachment ability of bacteria cultivated in TY medium enriched with two different glucose concentrations was compared with the attachment under

TABLE 1. Influence of extra glucose in the medium on growth, agglutination to glass, and cap formation of *R. leguminosarum* 248[a]

Additional glucose (mM)	A_{620} at end of log phase	Bacterial no. at end of log phase (10^9/ml)	Optimal A_{620} for cap formation
0	0.72	3.8	0.70
1	0.88	5.1	0.86
2	1.04	6.1	1.06

[a] Agglutination of cells to the glass was used as an indication for the end of the log phase.

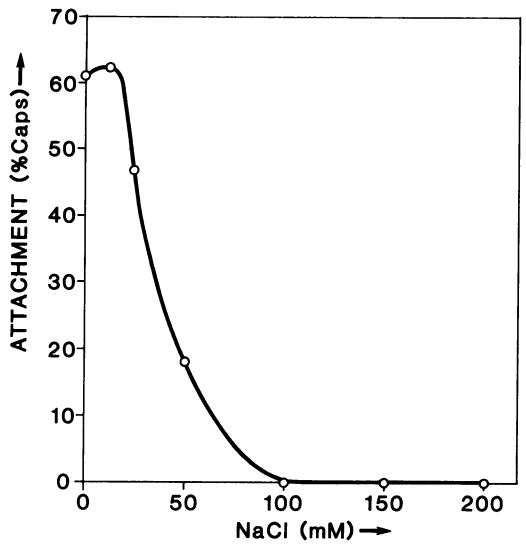

FIG. 4. Influence of sodium chloride on cap formation by *R. leguminosarum* 248 on pea root hair tips. The cells were harvested at an A_{620} value of 0.70. NaCl was added to the bacterial suspension just before the addition of the roots.

standard conditions, optimal attachment always coincided with the initiation of agglutination to glass and thus with C limitation (Table 1).

Effect of salts on cap formation. The presence of 25 mM NaCl during the attachment assay resulted in a strong decrease of attachment of *R. leguminosarum* 248, whereas a concentration of 100 mM abolished cap formation completely (Fig. 4). Other salts inhibited attachment similar to NaCl (Table 2). Incubation of plant roots or bacteria for 2 h in phosphate buffer supplemented with 100 mM NaCl just before the attachment assay did not result in an inhibition of attachment (data not shown).

Effect of haptenic sugars on cap formation. To examine the possible role of lectins in the attachment process, we tested a number of pea lectin haptens for inhibition of attachment of *R. leguminosarum* 248. Neither pea lectin haptens nor other sugars tested inhibited cap formation substantially (Table 3).

Host specificity. Various heterologous rhizobia were tested for their ability to adhere to pea root hairs. The bacteria were harvested when agglutination to glass started, which usually coincides with the late log phase. Fast-growing heterologous rhizobia appeared to attach almost equally as well to pea root hair tips as did *R. leguminosarum* cells, whereas *R.*

TABLE 2. Influence of various salts on the attachment of *R. leguminosarum* 248 to pea root hairs[a]

Salt added	Attachment (% in various classes)			
	1	2	3	4
None	0	26	8	66
NaCl	32	63	5	0
KCl	54	39	4	3
NH_4NO_3	28	66	4	2
$MnSO_4$	55	45	0	0
$MgSO_4$	37	62	0	1
$CaCl_2$	64	33	3	0

[a] Bacterial cells were harvested at an A_{620} value of 0.70, suspended in 25 mM phosphate buffer (pH 7.5), and incubated for 2 h with the roots. Salts were added to the bacterial suspensions just before the addition of the roots, in a final concentration of 100 mM.

TABLE 3. Influence of the addition of various sugars on the attachment of *R. leguminosarum* 248, harvested at an A_{620} value of 0.070[a]

Sugars added	Attachment (% in various classes[b])			
	1	2	3	4
None	1	25	1	73
α-D-Mannopyranoside[c]	0	38	5	57
3-*O*-Methyl-D-glucose[c]	1	26	10	63
D-Mannose[c]	1	24	15	60
1-*O*-Methyl-D-glucose	3	26	8	63
D-Glucose	1	25	5	69
D-Galactose	2	26	4	68
D-Xylose	0	17	3	80

[a] Sugars were added to the bacterial suspension just before incubation with the pea roots, in a final concentration of 50 mM.
[b] Class 4 attachment represents cap formation.
[c] Strong pea lectin hapten.

trifolii 5020 and 5523 adhered even more strongly (Table 4). It is interesting to note that *R. trifolii* 5020 and 5523 also autoagglutinated very strongly at every growth phase.

Extracellular fibrils. *R. leguminosarum* 248 produces extracellular fibrils, which can easily be distinguished from flagella since flagella have a diameter of 12 to 13 nm and are sinusoidal (Fig. 5A). The fibrils are exposed peritrichously on the bacteria and have a diameter of 5 to 6 nm and a length varying from 1 to over 10 μm (Fig. 5B). Fibrils do not occur very abundantly (1 to 10 per cell) and were mostly found in clumps of bacteria in which they cross bridged the distance between the cells. Rarely, a second type of fibril was observed with a diameter of approximately 4 nm and a length of 1 to 2 μm. As shown above, agglutinating bacteria adhere very well to pea root hair tips. Since agglutinated bacterial clumps were rich in fibrils, attempts were made to see whether fibrillation, adhesion to root hairs, and agglutination to glass are correlated.

R. leguminosarum 248 bacteria were harvested at various growth phases, and the percentage of fibrillated bacteria was estimated. A strong correlation between the occurrence of fibrillated bacteria and cap-forming ability was found (Fig. 3). Agglutination to glass was observed at the A_{620} value at which the percentage of fibrillated bacteria was optimal. Similar results were obtained for *R. leguminosarum* RBL1.

TABLE 4. Attachment of heterologous rhizobia to pea root hair tips[a]

Bacterium (A_{620})	Attachment (% in various classes)			
	1	2	3	4
R. leguminosarum 248 (0.700)	1	25	1	73
R. leguminosarum RBL1 (0.829)	4	26	28	42
R. trifolii 5020 (0.526)	0	3	5	92
R. trifolii 5523 (0.536)	0	3	3	94
R. trifolii 0403 (0.750)	8	43	14	35
R. phaseoli 1233 (1.019)	0	34	8	58
R. phaseoli RBL25 (0.400)	0	50	22	28
R. meliloti 1021 (1.251)	38	53	3	6
R. lupini M2 (0.219)	41	41	12	6
R. japonicum 784 (0.244)	66	25	8	1
R. japonicum RBL25 (1.428)	29	57	9	5

[a] Rhizobium cells were harvested at A_{620} values at which agglutination to glass started (these values are given in parentheses), suspended in phosphate buffer to a final concentration of 1.5×10^8 to 2.0×10^8 cells per ml, and incubated for 2 h with pea roots.

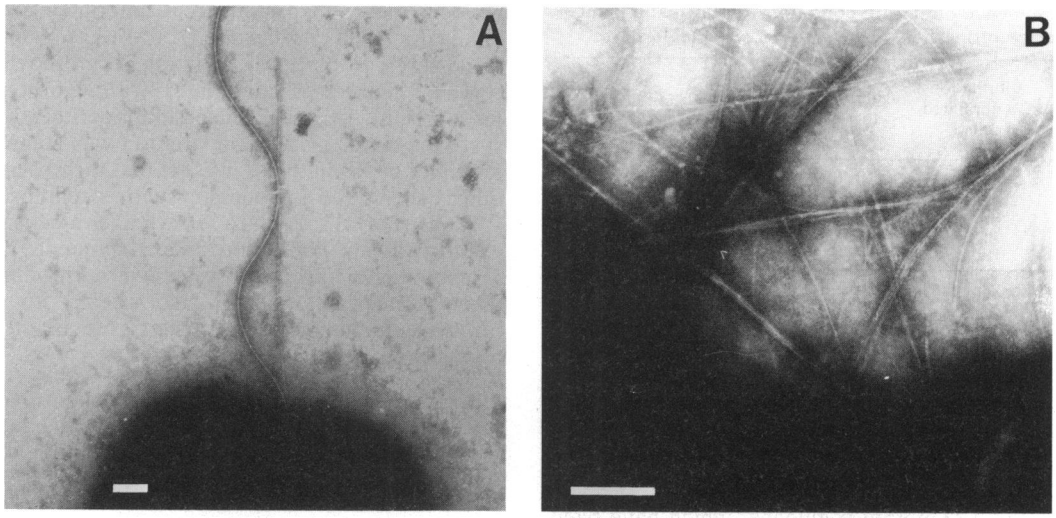

FIG. 5. Electron micrographs of fibrillated *R. leguminosarum* 248 cells. (A) Single fibril and a sinusoidal flagellum. (B) Several fibrils within a bacterial aggregate. Cells were negatively stained with a 1% phosphotungstic acid solution, pH 7.2. Bar, 200 nm.

R. leguminosarum bacteria were able to hemagglutinate human, calf, horse, and guinea pig erythrocytes. Hemagglutination was positively correlated with the percentage of fibrillated bacteria, and it was not affected by the addition of up to 50 mM mannose or galactose (data not shown).

Influence of pellicle growth on fibrillation and adherence. *R. leguminosarum* 248 formed a surface pellicle when cultivated in standing batch culture. In an attempt to increase the adherence properties of the rhizobia, bacteria from a surface pellicle were transferred three to five times to fresh unaerated medium. The final standing cultures were clear, and growth was only visible in the surface pellicle and as clumps at the bottom of the culture. The pellicle finally obtained was used as an inoculum for a vigorously aerated culture. In this case the bacteria were heavily fibrillated and showed excellent adherence to pea root hair tips, to glass, and to erythrocytes at much lower bacterial cell densities than bacteria from cultures inoculated from slants (Table 5).

Sym plasmid and attachment. To examine the possible involvement of Sym plasmidborne nodulation genes in the properties described above, the Sym plasmid-cured strains *R. leguminosarum* 248^c and *R. trifolii* 5039 were tested for the presence of fibrils and the ability to form a pellicle, to hemagglutinate, to agglutinate to glass, and to form caps. None of these characteristics was affected by loss of the Sym plasmid (data not shown).

DISCUSSION

As part of a program to study the components involved in nodulation, we started a detailed study of (one of) the first stage(s) of nodulation, namely, attachment of the *Rhizobium* bacteria to the plant root hair. Various grades of attachment were observed (Fig. 1). Since attachment to the tip of the root hair is supposed to precede the next infection step, marked root hair curling (30), we reasoned that cap formation (Fig. 1D) seemed to be the best criterion for successful adherence. It is conceivable that cap formation results in a relatively high concentration of a nodulation factor(s) involved in root hair curling (3) and root hair growth (25).

A lag time was observed before rhizobial caps were formed (Fig. 2). This lag time is presumably at least partly due to the fact that a minimal number of attached bacteria is needed before a cap can be observed. Alternatively, cap formation could be an autocatalytic process which first requires that a number of bacteria attach to the root hair before the process of cap formation, mainly owing to bacterial autoagglutination, begins. After approximately 90 min the maximal number of caps is observed. This does not necessarily imply that the bacteria stop adhering. It is conceivable that bacteria continue to adhere to other bacteria present in already existing caps.

Apart from the top of developing root hairs, bacteria also prefer to adhere to wound tissue and dead epidermal cells. This means that an attachment assay in which the number of attached bacteria is quantified with no regard to the site at which they adhere can be misleading when the results are connected with infection. The disadvantage of the present assay is that the number of bacteria attached at the root hair tip cannot easily be quantified.

One of the factors influencing cap formation is the pH of the buffer. Members of the family *Rhizobiaceae* are not acid tolerant (11). Since nodulation is inhibited in acid soil conditions (16), weak cap-forming ability of the bacteria at low soil pH may be (partly) responsible for this behavior.

Optimal cap formation was observed during the late log to early stationary phase (Fig. 3), when agglutination of the bacteria to glass started. Carbon was found to be the limiting growth factor, and optimal attachment ability of the rhizobia was correlated with carbon limitation and with agglutination to glass (Table 1).

Since it is known that sodium chloride inhibits nodulation of certain rhizobia (9, 23), the effect of NaCl and other salts on the attachment process was tested. The addition of sodium chloride or other salts to the incubation buffer in a final concentration of 100 mM or more abolished cap formation completely (Fig. 4 and Table 2). The results suggest that cap formation is based on electrostatic interactions. Alternatively, salts might detach adhesin from bacterium or plant. Since pretreatment of bacteria or plant roots with NaCl did not result in inhibition of cap formation, the first explanation, that cap formation is based on electrostatic interactions, seems most likely. Further research is required to clarify whether the inhibition of nodulation by NaCl is based on inhibition of attachment.

Lectin haptens added to the bacterial suspension just

TABLE 5. Influence of culture conditions on fibrillation and adherence properties of *R. leguminosarum* 248[a]

Mode of pregrowth of cells	Fibrillation (% fibrillated cells)[b]	Initiation of agglutination to glass (A_{620})	Hemagglutination[b]	Cap formation (% of class 4 attachment)[b]
Slopes with A^+ medium	25	0.70	−	23
Pellicle	50	0.40	+	60

[a] After pregrowth on slopes with A^+ medium (standard procedure) or as a surface pellicle derived from a standing culture (see text), bacteria were grown in TY medium, and various properties were tested.

[b] Fibrillation, hemagglutination, and cap formation were estimated at an A_{620} value of 0.40, representing the mid-log phase of growth.

before incubation failed to inhibit attachment of *R. leguminosarum* 248 (Table 3). If lectins were involved in cap formation, effective inhibition of cap formation, especially by D-mannose and 3-*O*-methyl-D-glucose, which have been shown to be strong pea lectin haptens (28), could be expected. Therefore, our results indicate lack of involvement of pea lectins under the tested conditions.

Attachment of *R. leguminosarum* to pea root hairs apparently is not a host-specific process since heterologous fast-growing rhizobia are able to adhere equally well (Table 4). *R. trifolii* 5020 and 5523 adhered even better than *R. leguminosarum*. Both lack of inhibition by pea lectin haptens and good attachment of Sym plasmid-cured and heterologous rhizobia do not support the lectin recognition hypothesis as an explanation of host-specific attachment. Preliminary results show that *R. leguminosarum* 248 bacteria also adhere well to *Phaseolus* root hairs. These results indicate that expression of host specificity occurs in one of the following steps in the infection process, e.g., infection thread initiation.

It is important to note that heterologous rhizobia did not always adhere at the same A_{620} value at which *R. leguminosarum* 248 adheres optimally (Table 4). Again, optimal attachment was found when the bacteria started agglutinating to glass. The differences in attachment optimum reflect growth characteristics and might explain differences found in attachment ability between various fast-growing rhizobia as described by others who compared attachment of various rhizobia at the same A_{620} value (4). Slow-growing heterologous rhizobia did not adhere as well as *R. leguminosarum* (Table 4). This might point to a common adhesion factor among fast-growing rhizobia which is absent or produced in a much lower amount by slow-growing species.

R. leguminosarum appears to produce extracellular fibrils (Fig. 5). The degree of fibrillation is strongly dependent on the growth phase (Fig. 3), and repeated growth as a pellicle strongly increases fibrillation. Variation of growth conditions influences a number of bacterial properties in a similar way. These properties are fibrillation, cap formation (Fig. 3), adherence to glass, and hemagglutination (Table 5). Also, for *R. trifolii* 5523 a positive correlation between fibrillation, agglutination to glass, and cap formation was observed. *R. trifolii* 5523 produces more fibrils (data not shown) and had a stronger cap-forming ability than *R. leguminosarum* (Table 4). These results strongly suggest that extracellular fibrils are responsible for the various types of adherence.

The nature of the fibrils is not yet known, but a number of the described characteristics of the rhizobial cells fit within the hypothesis that they are fimbriae. Pellicle formation in standing culture and agglutination of the bacterial cells were also described for a number of fimbriated members of the family *Enterobacteriaceae* (13, 19). *R. leguminosarum* 248 cells were capable of agglutinating erythrocytes, like many types of fimbriated bacteria (13, 26; Korhonen et al., in press). If the fibrils are indeed fimbriae, they are not type 1, since the hemagglutination reaction was mannose resistant. It cannot be excluded, however, that the observed fibrils are other cell surface components such as cellulose microfibrils. It is our short-term aim to see whether a causal relationship exists between fibrillation and attachment, especially cap formation. For this purpose we will purify fibrils and study their properties. We will also attempt to isolate fibril-negative mutants to study their role in attachment and nodulation.

ACKNOWLEDGMENTS

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